P19771.P01

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	P19771	Total Pages	

Inventor(s) or Application Identifier
Susumu SEINO, Tadao SHIBASAKI, and Nobuaki OZAKI

Title: PROTEIN RIM2

ADDRESS TO: **Assistant Commissioner for Patents**
Box Patent Application
Washington, DC 20231

APPLICATION ELEMENTS	ACCOMPANYING APPLICATION PARTS
☒ 1. Fee Transmittal Form	☒ 8. Assignment Papers (cover sheet & document(s))
☒ 2. Specification [Total Pages 41] (preferred arrangement set forth below) - Descriptive title of the Invention - Cross References to Related Applications - Statement Regarding Fed sponsored R & D - Reference to Microfiche Appendix - Background of the Invention - Brief Summary of the Invention - Brief Description of the Drawings (if filed) - Detailed Description - Claim(s) - Abstract of the Disclosure	☐ 9. 37 CFR 3.73(b) Statement ☐ Power of Attorney (when there is an assignee)
	☐ 10. English Translation Document (if applicable)
	☐ 11. Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
☒ 3. Drawing(s) (35 USC 113) [Total Sheets 8]	☒ 12. Preliminary Amendment
☒ 4. Oath or Declaration [Total Pages 4] a. ☒ Newly executed (original or copy) ☐ Unexecuted b. ☐ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with Box 18 completed) **[Note Box 5 below]** i. ☐ DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).	☒ 13. Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
	☒ 14. Small Entity ☐ Statement filed in prior application, Statements(two) Status still proper and desired
	☐ 15. The prior application is assigned of record to _____.
	☐ 16. Foreign priority claimed a. ☐ Claim of Priority b. ☐ Certified Copy of Priority Document(s)
	☐ 17. Other: _____
☐ 5. Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	
☐ 6. Microfiche Computer Program (Appendix)	
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary) a. ☒ Computer Readable Copy b. ☒ Paper Copy c. ☐ Statement verifying identity of above copies	

18. **If a CONTINUING APPLICATION,** check appropriate box and supply the requisite information:
☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior Application No. _____/_____, filed _____.

19. ☐ Amend the specification by inserting before the first line the sentence:
This application is a __ continuation-in-part, __ continuation, __ division, of Application No. _____/_____, filed _____.

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Applicant or Patentee: Susumu SEINO et al. Serial or Patent No: _____
Attorney's Docket No.:_____
Filed or Issued: _____
For: Protein Rim2

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9 (f) and 1.27(c)) -- SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[V] an official of the small business concern empowered to act on behalf of the concern identified below:
NAME OF SMALL BUSINESS CONCERN JCR Pharmaceuticals Co., Ltd.
ADDRESS OF SMALL BUSINESS CONCERN 3-19 Kasuga-cho, Ashiya-shi, Hyogo 659-0021 Japan

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12 and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. for purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Protein Rim2 by inventor(s) Susumu SEINO et al., described in

[ ] the specification filed herewith
[ ] application serial no. _____, filed _____.
[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(3) *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Shin Ashida
TITLE OF PERSON IF OTHER THAN OWNER President
ADDRESS OF PERSON SIGNING JCR Pharmaceuticals Co., Ltd., 3-19 Kasuga-cho, Ashiya-shi, Hyogo 659-0021 Japan

SIGNATURE _____ DATE June 27, 2000

NF301.WPF - SES FORM SMALL BUSINESS CONCERN

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Applicant or Patentee: Susumu SEINO et al.
Serial or Patent No: _____ Attorney's Docket No.: _____
Filed or Issued: _____
For: Protein Rim2

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9 (f) and 1.27(b)) -- INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled Protein Rim2
described in

[ ] the specification filed herewith
[ ] application serial no. _____, filed _____.
[ ] patent no. _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9 (c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

[ ] no such person, concern, or organization
[✓] persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Susumu SEINO	Tadao SHIBASAKI	Nobuaki OZAKI
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Susumu Seino	Tadao Shibasaki	Nobuaki Ozaki
Signature of Inventor	Signature of Inventor	Signature of Inventor
June 29, 2000	June 29, 2000	June 28, 2000
Date	Date	Date

(NF300.WPF - SES FORM INDEPENDENT INVENTOR)

P19771.A01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Susumu SEINO et al.

Serial No : Not Yet Assigned

Filed : Concurrently Herewith

For : PROTEIN RIM2

PRELIMINARY AMENDMENT

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to calculation of the filing fees and the examination of the above-identified patent application on the merits, the Examiner is respectfully requested to amend the claims as follows:

IN THE CLAIMS

Please amend the claims as follows:

Claim 3, line 1, delete "or 2"

Claim 5, lines 3 and 4, delete "or 2"

Claim 10, line 2, change "one of claims 4 to 7" to ---claim 4---.

Claim 13, line 2, delete "or 2"

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REMARKS

By the above amendment, the claims have been amended to delete multiple dependency.

If there should be any questions, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,
Susumu SEINO et al.

Leslie J. Paperner Reg No 33,329
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July 14, 2000
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PROTEIN RIM2

FIELD OF THE INVENTION

The present invention relates to protein Rim2, which is a novel isoform of Rim, i.e., a protein that interacts with a low molecular G protein Rab3 and is proposed to serve as a regulator of Rab3-dependent synaptic vesicle fusion, and which specifically interacts with the GDP/GTP exchange factor (GEFII; a cAMP sensor). More specifically, the present invention relates to elucidation of the mechanisms of intracellular vesicle transport and secretion, and to the novel protein Rim2 which is useful in diagnosis of endocrine-related diseases or neuropathy and in development of agents for prevention and treatment thereof, the gene encoding Rim2 and an antibody addressed to Rim2 protein.

Rim2 is considered to be a regulatory factor of vesicle fusion. It was found in the course of the present invention that the protein is expressed predominantly in endocrine tissues and endocrine- and neuroendocrine-derived cell lines. GTP-Rab3/GEFII/Rim complex is thought to participate in the regulation of exocytosis of neurons and endocrine cells, in a cAMP-dependent and protein kinase A (PKA) independent manner.

BACKGROUND OF THE INVENTION

Transport of substances between cell organelles, which are unit membrane-enclosed structures such as endoplasmic reticulum, is conducted by intracellular vesicle transport. In endocrine cells including pancreatic β-cells and pituitary cells, peptides/proteins synthesized at ribosomes are received by the endoplasmic reticulum, from which they are transported in vesicles, which are transformed into secretory vesicles through the Golgi body and transported to the cell membrane, where they are released out of the cell via a step which includes fusion of the membranes. In neurons, neurotransmitter-containing precursors of synaptic vesicles are formed in Golgi bodies and transported by microtubules along the axon and stored at the synapse. Depolarization of the pre-synaptic membrane causes the vesicles to fuse with the pre-synaptic membrane and thus the neurotransmitters are released. This type of secretion based on the fusion of the vesicles and the cell membrane is called exocytosis.

In contrast, when extracellular substances such as hormones including cell

growth factors are bound to the cell membrane, the complexes thus formed are invaginated into the cell to form endosomes. This type of uptake of environmental substances is called endocytosis.

Formation of vesicles, such as by budding, commonly observed both in exocytosis and endocytosis, and docking and fusion, the phenomena observed in process of their transportation and binding to other membrane systems, are regulated by a GTP-binding, low-molecular protein, called G protein. More than 30 types of this protein are known. The group of the proteins, which are also classified in Rab family, regulate the intracellular vesicle transport system.

With regard to the intracellular vesicle transport system, it is understood today that a cell is in a resting state when Rab protein occurs in a bound form to guanine nucleotide diphosphate (GDP), and that budding, docking and fusion are triggered as a result of a process in which a protein having GEF activity act on Rab protein and converts it to GTP-binding Rab protein, to which GTP binds to form a GTP-Rab complex, which in turn binds to a corresponding target protein on the membrane.

Stimulus-secretion coupling plays an important role in exocytosis observed in many cell types including neurons and endocrine cells [J.E. Rothman, Nature 372:55(1994); T.C. Sudhof, Nature 375:645 (1995)]. While a rise in intracellular Ca^{2+} concentration is important in the regulation of exocytosis, other signals are also known to play important roles. cAMP (cyclic adenosine-3',5'-monophosphate)/PKA (cAMP-dependent protein kinase A) signaling pathway is known to regulate exocytosis in many of neurons, neuroendocrine cells and endocrine cells. In particular, cAMP has been thought to mediate long-term potentiation by increasing neurotransmitter release in the brain [R.D. Hawkins et al. Ann. Rev. Neurosci. 16:625(1993); G. Lonart et al., Neuron 21:1141(1998)]. cAMP also regulates exocytosis responsible for insulin release from pancreatic β-cells and amylase release from parotid acinar cells [P.M. Jones and S.J., Persaud, Endocrine. Rev. 19:429(1998); E. Renstrom, et al., J. Physiol. 502:105(1997); K. Yoshimura, Biochim. Biophys. Acta 1402:171(1998)].

In addition to its role in PKA-dependent phosphorylation of regulatory proteins associated with the process of exocytosis, it is known that cAMP also acts directly on the exocytotic machinery in neurons and non-neuronal cells [G. Lonart et al., Neuron 21:1141 (1998); E. Renstrom et al., J. Physiol. 502:105 (1997); K.

Yoshimura, Biochim. Biophys. Acta, 1402:171(1998)].

During the search by the yeast two-hybrid screen (i.e., a method for detection of the interaction between two proteins in yeast cells) for an intracellular signaling molecule directly coupling to a sulphonylurea receptor, a component of pancreatic β-cell ATP-sensitive K^+ (K_{ATP}) channels [N. Inagaki et al. Proc. Natl. Acad. Sci. U.S.A. 91,2679 (1994)], a cAMP sensor protein (called "CAMPS") was identified and it was found that the protein has two putative cAMP binding domains, a Pleckstrin homology domain (PH domain), and a guanine nucleotide exchange factor (GEF) homology domain.

In the course of this study, two study groups independently reported cAMP binding proteins that activate Rap1, a member of the small G binding proteins [J. de Rooiji et al. Nature 396:474 (1998); H. Kawasaki et al. Science 282:2275 (1998)], and CAMPS was incidentally revealed to be a mouse homologue of cAMP-GEFII [H. Kawasaki et al. Science 282:2275 (1998)].

Though the mechanisms of intracellular vesicle transport system have thus gradually been clarified, substantial part of them remains still unknown. Further progress is needed for the understanding of the mechanisms so as to provide diagnostic agents or therapeutics for a variety of diseases which involve neurons or endocrine cells.

Unlike the former suggestion that only a single cAMP binding domain was present in cAMP-GEFII, the study by the present inventors suggested the presence of two putative cAMP binding domains (cAMP-A and cAMP-B), based on a sequence alignment of cAMP-GEFII sequence and regulatory subunits of PKA. Figure 1 shows the sequence alignment of the cAMP binding domains. The cAMP binding domains A and B (cAMP-A and cAMP-B, respectively) of cAMP-GEFII and the cAMP binding domains A and B of the PKA regulatory subunit Iα (RIα-A and RIα-B, respectively) are shown. The invariant residues in the different cAMP-binding domains are indicated by black boxes.

As shown in Figure 2, a glutathione-S-transferase (GST)-cAMP-A fusion protein bound to [^{3}H]cAMP with a dissociation constant (Kd) of ~10μM, while the binding of [^{3}H]cAMP to a GST-cAMP-B fusion protein was not evident under the same conditions.

Figure 2 shows the binding of cAMP to cAMP-A. GST-cAMP-A (filled circles) or GST-PKA RIα (open circles) was incubated with different concentrations

of [^{3}H]cAMP (0-50 μM). The data for cAMP-A or PKA RIα are normalized relative to maximal cAMP binding activities. Kd values are 10.0±2.3 μM and 23.7±0.6 nM for cAMP and PKA RIα, respectively.

In the cAMP-B domain, the amino acid residue 423, which originally is glutamic acid (Glu), is substituted with lysine (Lys). This glutamic acid residue is important for cAMP binding. Considering that a more rapid dissociation than the wild-type was observed with a PKA regulatory subunit having an equivalent mutation (E-200-K), cAMP-B may also dissociate cAMP rapidly. Thus, a possibility remains that cAMP binds to the cAMP-B domain.

SUMMARY OF THE INVENTION

As identification of a target molecule of CAMPUS, cAMP-GEFII, would serve to show its physiological role, the present inventors attempted to find a molecule that interacts with cAMP-GEFII by means of a yeast two-hybrid screen (YTH) method on the MIN6 cDNA library (See "Identification of Interacting molecules by YTH Method").

Surprisingly, the present inventors found that cAMP-GEFII interacts with a novel isoform (named "Rim2" by the present inventors) of Rim (a molecule which specifically interacts with Rab3: Rab3-interacting molecule: Hereinafter referred to as "Rim1"). Rim1 protein is a putative effector of the small G protein Rab3 and is proposed to serve as a Rab3-dependent regulator of synaptic vesicle fusion [Y. Wang et al. Nature 388:593(1997)].

The full-length novel protein Rim2 sequenced by the present inventors, which consists of 1590 amino acid residues, was found to have 61.6 % identity with rat Rim1. As Figure 3 shows, a zinc finger, PDZ and two C2 domains were found highly conserved between Rim1 and Rim2.

Based on the above findings, the present invention provides a protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing.

The present invention further provides a protein having an amino acid sequence with one or more amino acids deleted, substituted, inserted or added relative to the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing and which has a property to interact with GDP/GTP exchange factor II.

The present invention further provides a mouse gene which encodes the

following proteins (1) or (2):

(1) a protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing,

(2) a protein having an amino acid sequence with one or more amino acids deleted, substituted, inserted or added relative to the above-identified amino acid sequence and which has a property to interact with GDP/GTP exchange factor II.

In the present specification, "one or more" amino acid residues are generally several (e.g., 3 or 4) to 10 residues.

The present invention further provides a DNA having a nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing, the DNA being a cDNA corresponding to the above protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing.

The present invention further provides a DNA having a nucleotide sequence with one or more nucleotides deleted, substituted, inserted or added relative to the nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing and encoding any one of the above proteins. Herein, "one or more" nucleotides are generally several (e.g., 3 or 4) to 10 nucleotides. A variety of such nucleotide sequences with one or more nucleotides deleted, substituted, inserted or added can be readily prepared by those skilled in the art by making use of the familiar knowledge on degeneracy of the genetic code.

The present invention further provides a DNA having the nucleotide sequence of the coding region of the any one of the above DNA's or of a DNA having the nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing.

The present invention further provides a DNA fragment consisting of a part of any one of the above DNA's.

The present invention further provides a probe comprising a DNA which hybridizes with the DNA consisting of any one of the above nucleotide sequences.

The present invention further provides a primer DNA fragment consisting of a partial sequence of any one of the above nucleotide sequences.

The present invention further provides a recombinant vector having any one of the above DNA's.

The present invention further provides a monoclonal or polyclonal antibody directed to any one of the above proteins.

The present invention further provides a diagnostic agent for human use

comprising any one of the above probes or antibodies. The diagnostic agent is useful in the test for such diseases as secretion disorders in secretory systems including pituitary, hypothalamus, pancreatic β-cells and parotid gland, or the test for brain-nervous system diseases.

The present invention further provides a therapeutic agent for any one of the above diseases.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a sequence alignment of the cAMP binding domains.

Figure 2 is a graph showing the binding of cAMP to cAMP-A.

Figure 3 illustrates a comparison of amino acid identity between Rim1 and Rim2, in zinc finger, PDZ and C2 domains.

Figure 4 shows the results of immunoblotting showing the interaction between cAMP-GEFII and Rim1 or Rim2.

Figure 5 shows the results of Northern blot analysis of cAMP-GEFII, Rim1 and Rim2 in various rat tissues and endocrine- and neuroendocrine-derived cell lines.

Figure 6 is the result of *In situ* hybridization showing the localization of Rim1 and Rim2 in mouse brain and pituitary.

Figure 7 is a graph showing the result of yeast two-hybrid assays.

Figure 8 illustrates the result of immunoblotting showing the interaction between Rab3A and Rim1 or Rim2 *in vitro*.

Figure 9 is a graph showing the time course for high K^+-induced GH secretion from PC12 cells cotransfected with GH and cAMP-GEFII.

Figure 10 is a graph showing the effect of forskolin on GH secretion from transfected PC cells.

Figure 11 is a graph showing forskolin-induced GH secretion from PC12 cells transfected with various mutant cAMP-GEFII.

Figure 12 is a graph showing the effect of H-89 on forskolin-induced GH secretion from PC12 cells transfected with cAMP-GEFII.

Figure 13 is a schematic illustration showing a model for cAMP-dependent exocytosis.

DETAILED DESCRIPTION OF THE INVENTION

A variety of mutants can be provided by means of recombinant DNA technology. First, mutations can be introduced into a DNA clone fragment through different chemical and/or enzymatic processes, and the mutant DNA's thus obtained are then sequenced to select particular mutants with intended merits. This method allows a systematic preparation of different mutants regardless of their phenotypes. General methods of preparing a mutant clone DNA are as follows.

1. With the help of an oligonucleotide, substitution, deletion, insertion or addition can be directly effected in a given DNA sequence. This method enables to introduce a number of mutations in a small region of a given DNA.

2. By using longer oligonucleotides, it is possible to synthesize a desired gene.

3. By means of region-specific mutagenesis, a desired mutation can be introduced into a large (1-3 kb) DNA region.

4. Linker-scanning mutagenesis of DNA is a method suited for introducing a cluster point mutation into a relatively small (4-10 bp) DNA region.

5. PCR is also utilized as a method for direct introduction of a mutation.

[References: Current Protocols in Molecular Biology., 3 Vols., Edited by Ausubel F.M. et al., John Wiley & Sons, Inc., Current Protocols., Vol. 1, Chapter 8: Mutagenesis of Cloned DNA, pages 8.0.1-8.5.10]

Also well known to those skilled in the art are methods of preparing plasmids or vectors which can express a desired gene including different mutations obtained by the above methods. That is, by inserting a DNA carrying a desired gene into a expression vector DNA using a combination of restriction enzymes and a ligase, a recombinant plasmid is readily constructed which carries the desired gene. The recombinant plasmid thus obtained is then introduced into different cells to transfect them, thereby producing transformed cells. Cells which may be utilized range from prokaryotes, e.g. *E. coli*, to yeast, insect, plant and animal cells. [References: Vectors Essential Data. Gacesa P. and Ramji D.P., 166 pages. BIOS Scientific Publishers Limited 1994., John Wiley & Sons in association with BIOS Scientific Publishers Ltd. Expression vectors, pages 9-12.]

Introduction of a recombinant plasmid into host cells is effected by calcium chloride method or electroporation. Calcium chloride method provides efficient transformation without requiring any special apparatus. For higher efficiency,

electroporation is recommended.

[References: Current Protocols in Molecular Biology, 3 Vols. Edited by Ausbel F.M. et al., John Wiley & Sons, Inc., Current Protocols, Vol. 1, unit 1.8: Introduction of Plasmid DNA into Cells, pages 1.8.1-1.8.10]

Two types are known of transfection generally carried out on animal cell lines, i.e., transient and permanent types. In transient transfection, transformed cells are cultured for 1 - 4 days to effect transcription and replication of the transfected gene, and then the cells are harvested and their DNA analyzed. Alternatively, in many studies, a stable transformant cell line is produced, in which the transfected gene is incorporated into the chromosomes. Examples of the method for transfection include calcium phosphate method, electroporation, and liposome fusion method.

[Reference: Current protocols in molecular biology. 3 vols. Edited by Ausubel F.M. et al., John Wiley & Son, Inc., Current Protocols. Vol. 1, chapter 9: Introduction of DNA into mammalian cells, pages 9.0.1-9.17.3.]

Polyclonal and monoclonal antibodies directed to the proteins (polypeptides) coded by Rim2 gene of the present invention or their fragments and analogues as well, are readily prepared using techniques well known in the art. Antibodies obtained may be used as laboratory reagents and diagnostic agents for diseases associated with Rim2 gene. The antibodies obtained are also used for preparation of antibody columns, for immunoprecipitation as well as for identification of the antigen by Western blotting.

A general method for preparing a monoclonal antibody in mg-scale directed to the proteins coded for by Rim2 gene of the present invention is as follows: Mice are inoculated with the antigen protein to immunize. The spleen is removed from the mice exhibiting a sufficient antibody titer. The spleen cells are dissociated, and selected B cells are fused with myeloma cells of B cell origin to form hybridoma cells which secrete the antibody. The monoclonal antibody secreted from the hybridoma cells is purified from the culture medium using an affinity column, ion-exchange, or gel filtration, etc. The polyclonal antibody of the present invention may be prepared by a conventional method: Using rabbits, horses, mice or guinea pigs as immunized animals, the antigen protein is inoculated along one of the schedules known in the art to immunize the animals, and then IgG, etc. are isolated from the collected serum.

[Reference: Current protocols in molecular biology, 3 vols. Edited by Ausubel F.M. et al., John Wiley & Sons, Inc., Current Protocols, Vol. 2, chapter 11: Immunology, pages 11.0.1-11.16.13.]

In order to assess the specificity of interaction between cAMP-GEFII and Rim2, the present inventors evaluated the binding of a FLAG-tagged cAMP-GEFII protein to a GST-Rim2 fusion protein immobilized on glutathione beads (See "Study on Interaction between Rim2 and cAMP-GEFII: I").

Briefly, lysates from COS-1 cells transfected with FLAG-tagged cAMP-GEFII, from MIN6 cells or from mouse brain homogenate were evaluated for binding to GST-Rim1, GST-Rim2 or GST alone. cAMP was detected by immunoblotting with an anti-FLAG antibody (Figure 4, left) or an anti-cAMP-GEFII antibody (Figure 4, center and right), respectively. These results demonstrates that cAMP-GEFII protein interacts with GST-Rim2 protein. Likewise, GST-Rim1 protein also bound to cAMP-GEFII in the mouse brain homogenate (See "Study on Interaction between Rim1 and cAMP-GEFII") (Figure 4, right). These results confirms that cAMP-GEFII interacts with Rim1 and Rim2.

Figure 5 shows the results of the northern blot analyses of cAMP-GEFII, Rim1 and Rim2 in various rat tissues and endocrine- and neuroendocrine-derived cell lines (See "Northern Blotting in Rat Tissues"). Ten μg samples of total RNA from various tissues and cell lines (except 5 μg for pancreatic islets) were used. Hybridization and washing were performed under standard conditions. The faint signals seen in Rim2 mRNA blot analysis of cerebrum and cerebellum are due to cross-hybridization with the Rim1 cDNA probe used. Figure 5 shows that Rim2 mRNA is expressed predominantly in endocrine tissues and endocrine- and neuroendocrine-derived cell lines, including pituitary, pancreatic Langerhans' islet cells, MIN6 cells, and PC12 cells. Rim2 mRNA was detected in the brain by reverse transcriptase-PCR (data not shown). Rim1 mRNA, in contrast, was found to be expressed in cerebrum, cerebellum, and pituitary by a similar analysis.

The major transcripts for Rim1 and Rim2 have 6.4 kb for Rim1, and 7.2 kb and 5.4 kb for Rim2. There are also found several minor transcripts, which occur due probably to alternative splicing.

cAMP-GEFII mRNA is generally coexpressed with Rim1 or Rim2 mRNA in tissues and cell lines in which regulated exocytosis is known to occur. Figure 6 illustrates the results of *in situ* hybridization showing the localization of Rim1 and

Rim2 in mouse brain and pituitary. In the figure: (a) cAMP-GEFII; (b) Rim1; (c) Rim2; (d) pituitary. The scale bar corresponds to 1 mm. Abbreviations: Cb = cerebellum, Cp = caudoputamen, Cx = cortex, Hi = hippocampus, Ob = olfactory bulb, Po = pons, Th = thalamus

Rim2 mRNA is found expressed only in the cerebellar cortex, while Rim1 mRNA is expressed in cerebral cortex, hippocampus (especially CA3 and dentate gyrus), olfactory bulb, and cerebellar cortex (See "*In situ* Hybridization in Mouse Brain"). The distribution of cAMP-GEFII mRNA overlaps largely with that of Rim1 mRNA in the brain. It is confirmed that Rim2 mRNA and cAMP-GEFII mRNA are coexpressed in anterior pituitary.

Rim1 is proposed to be a Rab3 effector, a low molecular weight G protein [Y. Wang, et al., Nature 388,593 (1997)]. Using yeast two-hybrid assays (See "Study on Interaction between Rim2 and Rab3A".), the present inventors found that Rim2, like Rim1, interacts with active Rab3A (Q81L) (Figure 7). Figure 7 shows the results of the yeast two-hybrid assays. Rim1, Rim2 or rabphilin3 and wild-type Rab3A or constitutively active Rab3A (Q81L) in various combinations were determined by transactivation of liquid β-galactosidase activity.

In addition, the immobilized GST-Rim2 bound only to the GTPγS-bound form of Rab3A (Figure 8). Figure 8 shows the interaction between Rab3A and Rim1 or Rim2 in vitro, which is the result obtained by incubating GTPγS- or GDP γS-bound form of Rab3A with GST-Rim1 (residues 1-201) and GST-Rim2 (residues 1-345) immobilized on glutathione beads, respectively. Rab3A was detected by immunoblotting with anti-Rab3A antibody. These results indicate that Rim2, like Rim1, binds to the GTP-activated form of Rab3A.

The interaction of cAMP-GEFII and Rim2 protein strongly suggests that cAMP-GEFII is involved in regulated exocytosis. To determine its functional role, the present inventors examined the effect of cAMP on $Ca2^{+}$-dependent secretion in PC12 cells cotransfected with growth hormone (GH) and cAMP-GEFII (See "Study on GH secretion from Transfected PC12 Cells").

Since PC12 cells endogenously express Rim2 but not cAMP-GEFII, the exogenously introduced cAMP-GEFII may form a complex with endogenous Rim2.

Figure 9 is a graph showing the time course of high K^{+}-induced GH secretion from PC12 cells cotransfected with GH and cAMP-GEFII. Figure 10 is a graph showing the effect of forskolin on GH secretion from the transfected PC12

cells. Forskolin (50 μM) was added 10 min before the incubation with a low K^+ (4.7 mM) or high K^+ (60 mM) solution. The meaning of the symbols are as follows: For basal (low K^+-induced) secretion: cAMP-GEFII-transfectant (filled triangles); β-galactosidase-transfectant (control)(open circles); high K^+-induced secretion: cAMP-GEFII-transfectant (filled circles); β-galactosidase-transfectant (control)(open circles). The values represent the percent GH amounts released into the medium relative to the total cellular GH amounts.

In the cotransfected PC12 cells, as shown in Figure 9, cAMP-GEFII did not alter Ca^{2+}-dependent (60 mM K^+) secretion of cotransfected GH, compared to the control, but significantly enhanced forskolin (50 μM)-induced, Ca^{2+}-dependent GH secretion (Figure 10). Forskolin acts mainly on adenylate cyclase, serving to increase cAMP levels in the cells. cAMP-GEFII also enhanced 8-Br-cAMP (1 mM)-induced, Ca^{2+}-dependent GH secretion (cAMP-GEFII-transfectant, 34.9 ± 1.3 %; control, 25.1 ± 1.8 %, n=9, P<0.001).

Figure 11 is a graph showing forskolin-induced GH secretion from PC12 cells transfected with various mutant cAMP-GEFII, in which the increment of forskolin (50 μM)-induced GH secretion (in the presence of high K^+) above the basal level during a 15-min incubation for each mutant cAMP-GEFII is expressed as percentage relative to the wild-type cAMP-GEFII (100%). In the figure: WT = wild-type cAMP-GEFII, T810A = mutant cAMP-GEFII (T810A); G114E, G422D = double mutant cAMP-GEFII (G114, G422D).

The forskolin-induced GH secretion was not affected in the mutant cAMP-GEFII (T810A) in which a potential PKA phosphorylation site is disrupted by substitution of one of its amino acids (Figure 11). In addition, the forskolin-induced GH secretion in the mutant cAMP-GEFII (G114E, G422D) in which both of the cAMP binding sites are disputed was reduced to ~40 % of that in the wild-type.

These results indicate that cAMP promotes Ca^{2+}-dependent GH secretion by binding to cAMP-GEFII, without involving its phosphorylation by PKA.

Figure 12 is a graph showing the effect of H-89 on forskolin-induced GH secretion from cAMP-GEFII-transfected PC12 cells. H-89 (10 μM) was added to the incubation buffer 10 min before forskolin (50 μM) treatment. The treatment with H-89 (10 μM) reduced high K^+-induced GH secretion in both of the cAMP-GEFII-transfected and β-galactosidase-transfected PC12 cells. The data were obtained from 3-5 independent experiments (A-D). The values are means ± SEM

(P<0.01).

Importantly, the forskolin-induced, $Ca2^{+}$-dependent GH secretion from the cAMP-GEFII-transfected PC12 cells treated with the PKA inhibitor H-89 was significantly higher than that from the control cells. This indicates that cAMP-GEFII mediates cAMP-dependent and PKA-independent exocytosis.

To ascertain the physiological relevance of cAMP-GEFII, the present inventors investigated the role of endogenous cAMP-GEFII in secretion. In insulin secretion from pancreatic β-cells, cAMP is proposed to stimulate exocytosis by PKA-dependent as well as PKA-independent mechanisms [M. Prentki, F.M. Matschinsky, Physiol. Rev. 67:1185 (1987)/ P.M.Jones, S.J. Persaud, Endocrine. Rev. 19:429 (1998)].

In the high glucose condition of 16.7 mM, 8-Br-cAMP-induced insulin secretion from MIN6 cells treated with antisense oligonucleotides against cAMP-GEFII was significantly reduced (87.5 ± 2.3 % of the secretion from MIN6 cells treated with a control oligonucleotide, n=27, P<0.005) (See "Study of the Role of cAMP-GEFII in cAMP-dependent Exocytosis"), suggesting that cAMP-GEFII participates in cAMP-dependent exocytosis in native cells.

Rab3 is associated with the final step of exocytosis. The structurally-related proteins rabphilin3 [H. Shirataki et al., Mol. Cell. Biol. 13,2061 (1993)] and Rim1 both bind to Rab3A, suggesting that multiple Rab3A effectors could operate in triggering docking and fusion of the vesicles to the plasma membrane.

In the process toward the present invention, it was found that the cAMP sensor, cAMP-GEFII, mediates cAMP-induced, Ca^{2+}-dependent exocytosis by interacting with a Rab3 effector Rim2.

In addition to its role in PKA phosphorylation of proteins associated with secretory processes, previous studies have suggested that cAMP may act directly on the exocytosis [G. Lonart, et al., Neuron 21:1141 (1998); E. Renstrom, et al., J. Physiol. 502:105(1997); K. Yoshimura et al., Biochim. Biophys. Acta 1402:171(1998)]. In pancreatic β-cells, too, PKA-dependent as well as PKA-independent stimulation of insulin release by cAMP has been proposed [E. Renstrom, et al., J. Physiol. 502:105 (1997)]. It is thought that cAMP probably directly stimulates amylase release in parotid acinar cells [G. Lonart, et al., Neuron 21:1141 (1998)]. In addition, a recent study suggests that cAMP enhances glutamate release in the brain partly by a direct action on the exocytotic machinery

[G. Lonart, et al., Neuron 21,1141 (1998)].

However, while both rabphilin3 and Rim1 are ubiquitously expressed in most of the synapses in the brain[C. Li et al., Neuron 13:885 (1994)], cAMP-enhanced glutamate release occurs in synaptosomes from the CA3 region in the hippocampus, not from the CA1 region, a finding consistent with cAMP-GEFII and Rim1 being coexpressed predominantly in CA3.

Accordingly, it is considered that, in addition to PKA-dependent phosphorylation in the secretory processes, cAMP promotes regulated exocytosis in a PKA-independent manner by acting directly on a complex of cAMP-GEFII (a cAMP sensor) and Rim (a Rab3 effector) in some neurons and neuroendocrine and endocrine cells, as schematically illustrated in Figure 13.

These findings indicates that Rim2 of the present invention also plays an important role in the regulation of exocytosis in neurons and endocrine cells.

EXAMPLES

The present invention will be described below in further detail by presenting specific procedures in the present invention with reference to an example.

<Sequencing of CAMPS (cAMP-GEFII) cDNA>

A plasmid cDNA library has been made from a mouse insulin-secreting cell line, MIN6, in the vector pVP16. A yeast two-hybrid bait vector was constructed in plasmid pBTM116 using a DNA fragment encoding partial rat SUR1 (amino acid residues 598-1003)(GenBank accession number L40624), a subunit of the pancreatic β-cell K_{ATP} channel.

Yeast two-hybrid screen of the plasmid MIN6 cDNA library was performed as described in K. Kotake et al., J. Biol. Chem. 272:29407 (1997). A prey clone encoding a partial CAMPS, a cAMP sensor, (residues 187-730) was isolated. A full-length mouse CAMPS cDNA was obtained from the λ MIN6 cDNA library [N. Inagaki et al., Proc. Natl. Acad. Sci. U.S.A. 91:2679(1994)]. The nucleotide sequence of mouse CAMPS (cAMP-GEFII) has been deposited in Genbank with the accession number of AB021132.

<Preparation and Test of GST fusion Protein>

cAMP-A (amino acid residues 43-153), cAMP-B (amino acid residues 357-469), and rat PKA regulatory subunit (RIα)(full-length) were expressed as GST-

fusion proteins using pGEX-4T-1 (Amersham-Pharmacia) and purified according to the manufacturer's instructions. cAMP binding assay was performed as described in R.A. Steiberg, et al., J. Biol. Chem. 262:2664(1987) with slight modifications.

Briefly, GST-fusion protein (1 μg) was incubated in binding buffer (200 μl) containing various concentrations of [^{3}H]cAMP, 50 mM potassium phosphate buffer (pH 6.8), 150 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.5 mg/ml bovine serum albumin with or without 40 mM unlabeled cAMP for 2 hrs on ice.

<Identification of Interacting molecules by YTH Method>

Yeast two-hybrid bait vector was constructed in plasmid pBTM116 using a full-length mouse cAMP-GEFII cDNA. A prey clone encoding a partial sequence of Rim2 (amino acid residues 53-863) was isolated from the plasmid MIN6 cDNA library. A full-length cDNA for Rim2 was obtained from the λ MIN6 cDNA library.

<Study on Interaction between Rim2 and cAMP-GEFII: I>

Rim2 (amino acid residues 538-863) was expressed as a GST fusion protein and purified according to the method described in "Preparation and Test of GST fusion Protein". A full-length cAMP-GEFII cDNA was subcloned into plasmid pFLAG-CMV-2 (Sigma). The resultant construct was transfected into COS-1 cells, using LipofectAMINE (Life Technologies). The lysate of the COS-1 cells was incubated with GST-Rim2 immobilized on glutathione beads for 2 hrs at 4℃. The complex thus obtained was washed with distilled water, separated by SDS-PAGE, and immunoblotted with an anti-FLAG M2 antibody (Sigma).

<Study on Interaction between Rim2 and cAMP-GEFII: II>

The lysate of MIN6 cells was incubated with GST-Rim2 and interaction between cAMP-GEFII and Rim2 was evaluated according to the method described in "Study on Interaction between Rim2 and cAMP-GEFII: I", using a IgG antibody raised against the C-terminus (amino acid residues 1001-1011, Gln-Met-Ser-His-Arg-Leu-Glu-Pro-Arg-Arg-Pro) (SEQ ID NO:5) of mouse cAMP-GEFII.

<Study on Interaction between Rim1 and cAMP-GEFII>

According to the method described in "Preparation and Test of GST fusion Protein", Rim1 partial sequence (530-806) was expressed as a GST fusion protein and then purified. The brain homogenate from three mice was incubated with GST-Rim1 immobilized on glutathione beads overnight at 4℃. cAMP-GEFII was detected as described in "Study on Interaction between Rim2 and cAMP-GEFII: II".

<Northern Blotting in Rat Tissues>

Northern Blotting was performed for various tissues of rat using, as probes, mouse cAMP-GEFII (nucleic acids 606-2237), rat Rim1 (1035-1491), and mouse Rim2 (586-1490) cDNA.

<*In situ* Hybridization in Mouse Brain>

In situ hybridization in mouse brain was performed as described in J. Tanaka, M. Murate, C.Z. Wang, S. Seino, T. Iwanaga, Arch. Histol. Cytol. 59:485 (1996).

Antisense oligonucleotide probes (45 mer) used for mouse cAMP-GEFII and Rim2 correspond to the regions of the nucleic acids 2746-2790 and 1376-1420, respectively.

For the antisense oligonucleotide for Rim1, Rim1 cDNA was partially cloned from mouse brain: the probe used in this was 5'-ttgcgctcactcttctggcctcccttgccattctgctctgaaagc-3' (SEQ ID NO:3).

<Study on Interaction between Rim2 and Rab3A>

According to the method described in "Identification of Interacting molecules by YTH Method", the full-length cDNA's for wild type mouse Rab3A and constitutively active bovine Rab3A (Q81L) were cloned into the yeast bait vector pBTM116.

The nucleotide sequence of zinc finger domains of bovine rabphilin3 (amino acid residues 1-283), rat Rim1 (amino acid residues 1-204) and mouse Rim2 (amino acid residues 1-345 were cloned into the prey vector pVP16. Liquid culture assay of β-galactosidase activities was performed according to the manufacturer's instructions (Clontech). The activity values were obtained from 3 independent clones for each transformant and normalized by cell numbers determined as OD_{600}.

Lipid-modified Rab3A was purified from the membrane fraction of Sf9 cells expressing Rab3A. Rat Rim1 (amino acid residues 1-204) and mouse Rim2 (amino acid residues 1-345) were expressed as GST fusion proteins and purified. The GTP γS- or GDPβS-bound form of Rab3A was incubated for 90 min at 4℃ with GST-Rim1, or GST-Rim2 (30 pmol for each) immobilized on glutathione beads in reaction buffer. Rab3A was detected by immunoblotting with anti-Rab3A antibody.

<Study on GH secretion from Transfected PC12 Cells>

GH secretion from transfected PC12 cells was performed as described in K. Korake et al., J. Biol. Chem., 272:29407(1997). Expression plasmid vectors (pSR

α) for wild-type cAMP-GEFII, mutant cAMP-GEFII (T810A), and the double mutant cAMP-GEFII (G114E, G422D) were prepared. As a control, β-galactosidase (β-gal) was used. PC cells were transfected with GH expression vector (pXGH5: Nichols Institute) plus each vector described above, using LipofectAMINE.

PC12 cell were incubated with a low K^+ (4.7 mM) or high K^+ (60 mM) solution, in the presence or absence of forskolin (50 μM) or 8-bromoadenosine 3',5' cyclic monophosphate (8-Br-cAMP)(1 mM). Forskolin or 8-Br-cAMP was added 10 min before the incubation with a low or high K^+ solution. In some experiments, the PKA inhibitor H-89 (10 μM) was added 10 min before forskolin stimulation.

<Study of the Role of cAMP-GEFII in cAMP-dependent Exocytosis>

To interfere with the synthesis of cAMP-GEFII in MIN6 cells, antisense phosphorothioate-substituted oligoDNA (16 mer) against mouse cAMP-GEFII (the region corresponding to nucleic acids 104-119) and control oligoDNA (5'-acctacgtgactacgt-3') (SEQ ID NO:4) were synthesized (BIOGNOSTIK).

MIN6 cells were treated with 4 μM of the antisense oligoDNA or control oligoDNA 24 hours before insulin secretion experiments. The efficacy of antisense oligoDNA was evaluated by immunoblot analysis of the antisense oligoDNA-treated MIN6 cells over-expressing cAMP-GEFII by transient transfection, using anti-cAMP-GEFII antibody. The level of cAMP-GEFII was markedly lowered in the antisense oligoDNA-treated MIN6 cells. Insulin secretory response to 8-Br-cAMP (1 mM) of these MIN6 cells was assessed in the presence of high glucose (16.7 mM). Five separate experiments were performed, in which insulin was measured as described in T. Gonoi et al., J. Biol. Chem. 269:16989 (1994).

SEQUENCE LISTING

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Gln Ile Ala Ser Asp Met Pro Ser Asp Arg Lys Arg Ser Pro Ser Val
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345 350 355

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Tyr Glu Arg Gln Arg Arg Glu Glu Glu Tyr Gln Ala Arg Tyr Arg Ser
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380 385 390

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1 5 10

WHAT IS CLAIMED IS:

1. A protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing.

2. A protein having an amino acid sequence with one or more amino acids deleted, substituted, inserted or added relative to the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing and which has a property to interact with GDP/GTP exchange factor II.

3. A mouse gene which encodes the protein of claim 1 or 2.

4. A DNA having a nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing, the DNA being a cDNA corresponding to the protein of claim 1.

5. A DNA having a nucleotide sequence with one or more nucleotides deleted, substituted, inserted or added relative to the nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing and encoding the protein of claim 1 or 2.

6. A DNA having the nucleotide sequence of the coding region of the DNA of claim 4.

7. A DNA having the nucleotide sequence of the coding region of the DNA of claim 5.

8. A DNA fragment consisting of a part of the DNA of claim 4.

9. A probe comprising a DNA which hybridizes with the DNA of claim 4.

10. A primer DNA fragment consisting of a partial sequence of the sequence of one of claims 4 to 7.

11. A recombinant vector having the DNA of claim 4.

12. A recombinant vector having the DNA of claim 5.

13. A monoclonal or polyclonal antibody directed to the protein of claim 1 or 2.

14. A diagnostic agent for secretory disorders or brain-nervous system diseases comprising the probe of claim 9.

15. A diagnostic agent for secretory disorders or brain-nervous system diseases comprising the antibody of claim 13.

ABSTRACT

Provided is a protein used in the development of a therapeutic agent for neuron- or endocrine cell-related diseases, in which the transport system is involved. The protein has an amino acid sequence with one or more amino acids deleted, substituted, inserted or added relative to the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing and which has a property to interact with GDP/GTP exchange factor II.

Figure 1

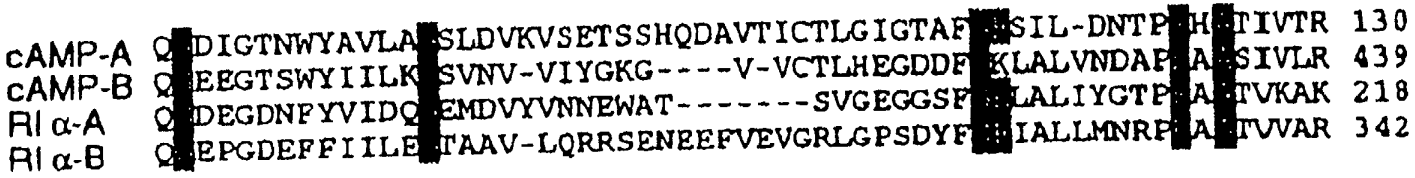
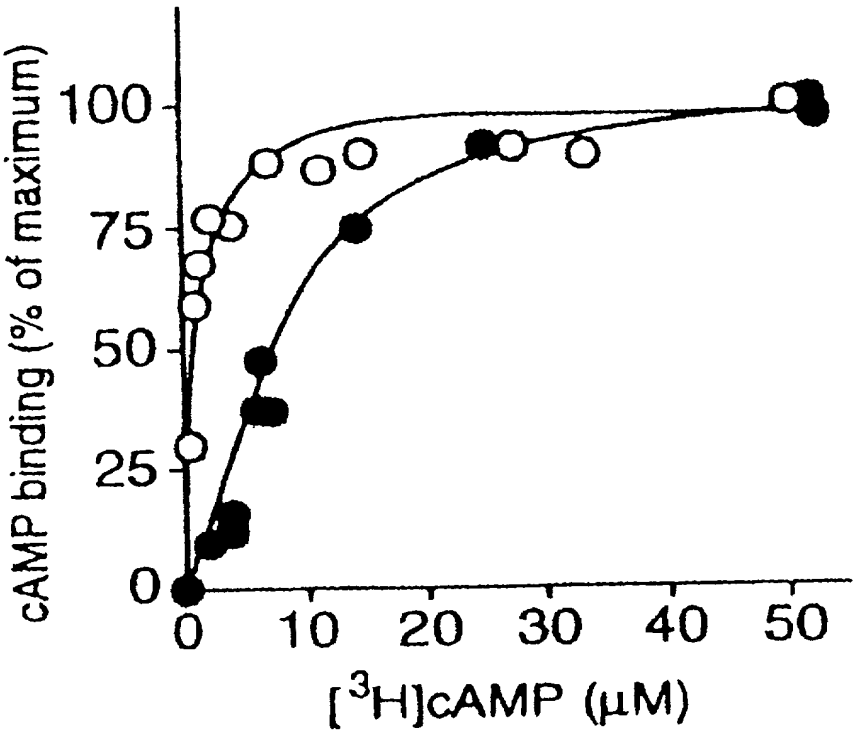


Figure 2



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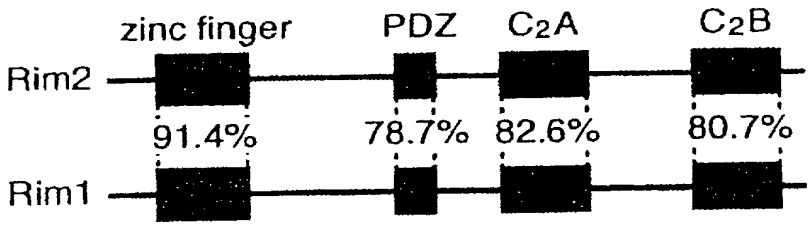

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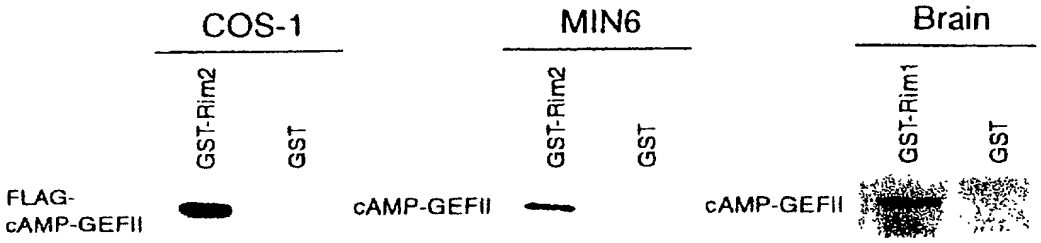
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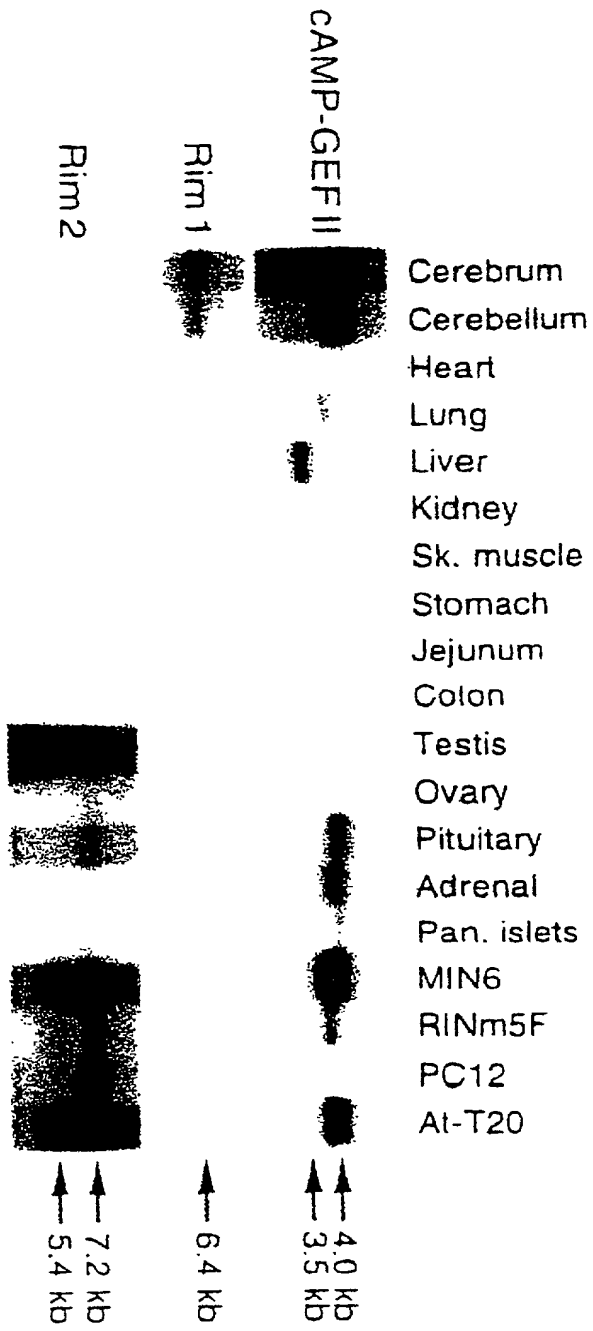
Figure 5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Stomach
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Testis
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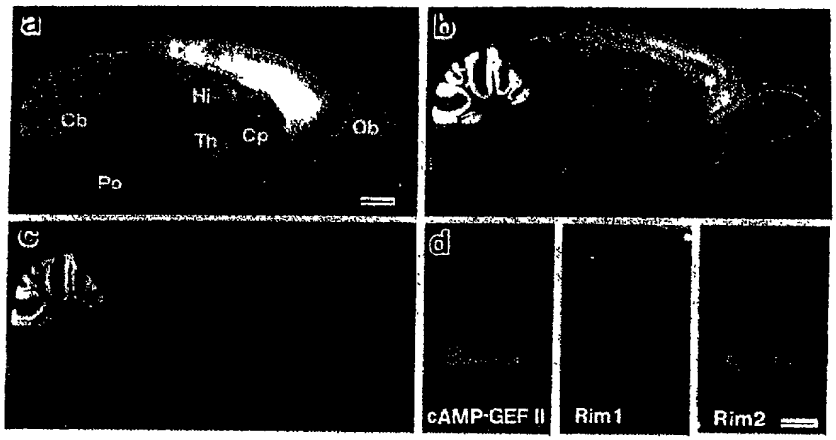
Figure 6



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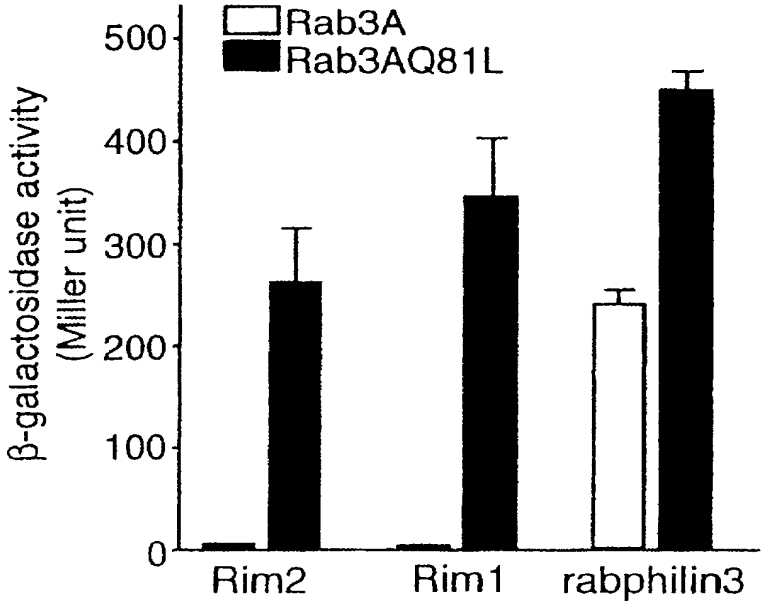

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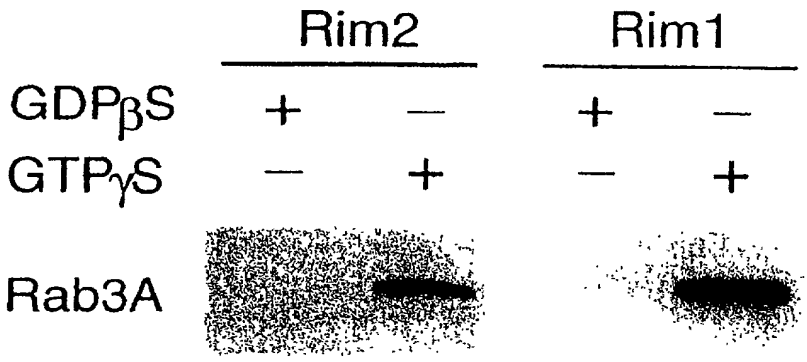

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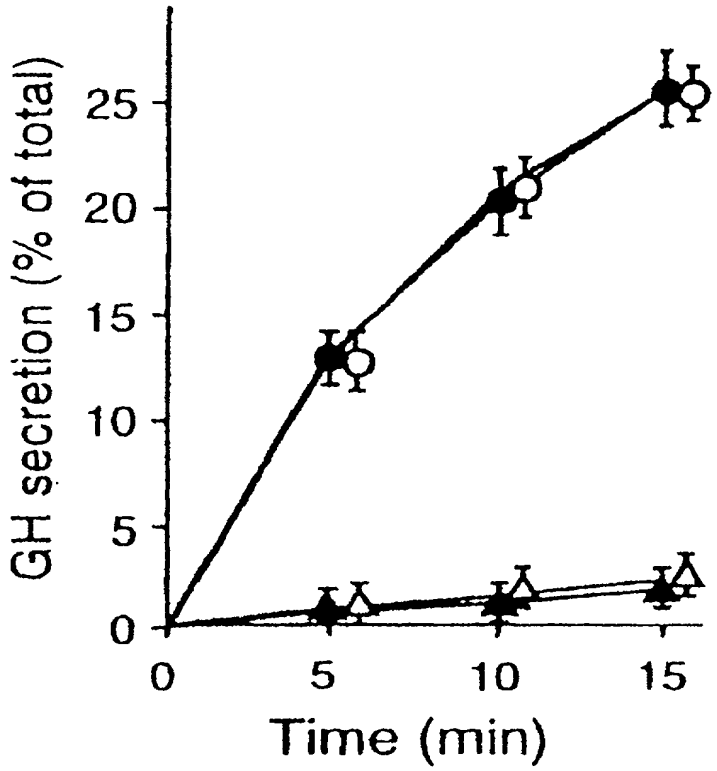

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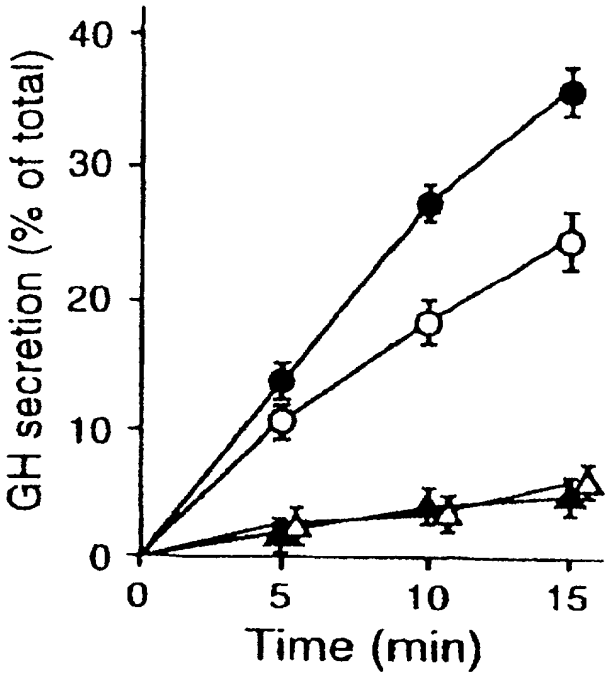

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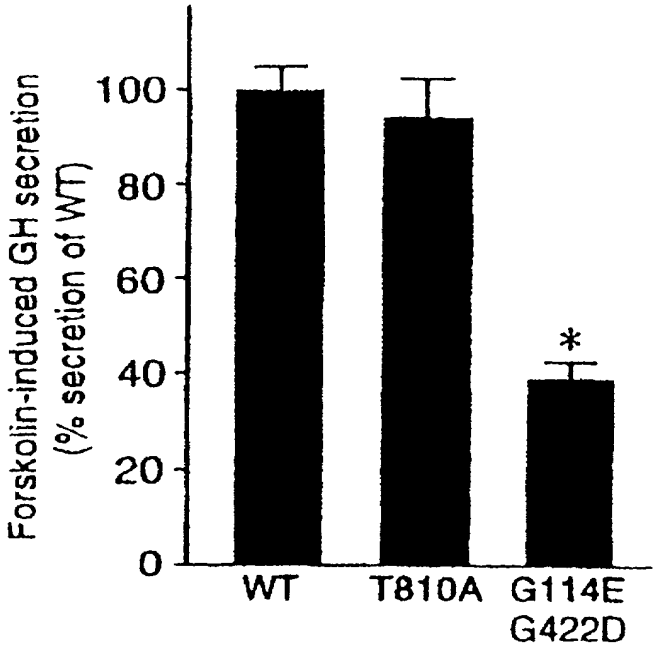

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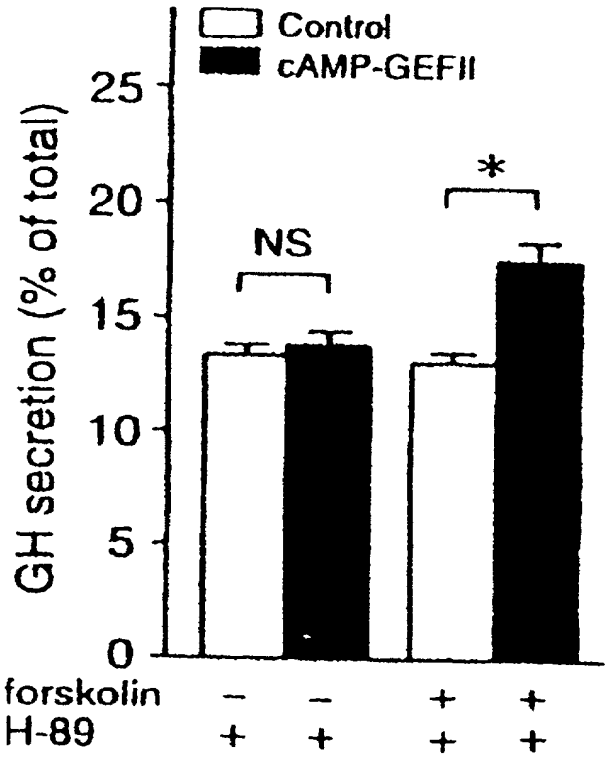
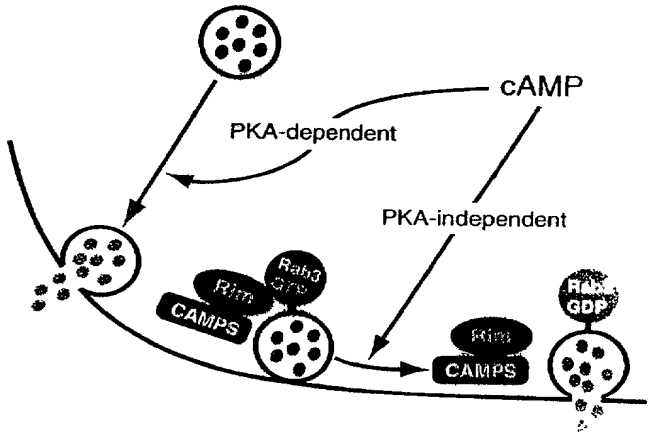


Figure 13



Declaration and Power of Attorney For Utility or Design Patent Application
特許出願宣言書

Japanese Language Declaration

私は、下欄に氏名を記載した発明者として、以下のとおり宣言する：

私の住所、郵便の宛先および国籍は、下欄に氏名に続いて記載したとおりであり、

名称の発明に関し、請求の範囲に記載した特許を求める主題の本来の、最初にして唯一の発明者である(一人の氏名のみが下欄に記載されている場合)か、もしくは本来の、最初にして共同の発明者である(複数の氏名が下欄に記載されている場合)と信じ、

_____

_____

_____

☐ その明細書を

(該当するほうに印を付す)

☐ ここに添付する。

☐ _____ 日に出願番号

第 _____ 号として提出し、

_____ 日に補正した。

(該当する場合)

☐ 私は、前記のとおり補正した請求の範囲を含む前記明細書の内容を検討し、理解したことを陳述する。

私は、連邦規則法典第37部第1章第56条に従い、本題の審査に所要の情報を開示すべき義務を有することを認める。

☐ 私は合衆国法典第35部第119条(a－d)項又は第365条(b)項に基づく、下記の外国特許出願又は発明者証出願、或いは第365条(a)項に基づく、少なくても米国以外の1ケ国を指名したPCT国際出願の外国優先権利益を主張し、更に優先権の主張に係わる基礎出願の出願日前の出願日を有する外国特許出願、又は発明者証出願或るいはPCT国際出願を以下に明記する：

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Protein Rim2

_____

_____

the specification of which

(check one)

☑ is attached hereto.

☐ was filed on _____ as

Application No. _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a-d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Prior foreign applications 先の外国出願			Priority claimed 優先権の主張	
288372 / 99 (Number) (番号)	JAPAN (Country) (国名)	08 / 10 / 1999 (Day/Month/Year Filed) (出願の年月日)	☑ Yes あり	☐ No なし
(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願の年月日)	☐ Yes あり	☐ No なし

☐ その他の外国特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

U.S. PTO Acceptance # 903

Japanese Language Utility or Design Patent Application Declaration

私は、合衆国法典第35部第119条(e)項に基づく、下記の合衆国仮特許出願の利益を主張する。

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

(Number) (番号)	(Day/Month/Year Filed) 出願の年月日
(Number) (番号)	(Day/Month/Year Filed) 出願の年月日
(Number) (番号)	(Day/Month/Year Filed) 出願の年月日

☐ その他の合衆国仮特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

私は、合衆国法典第35部第120条に基づく下記の合衆国特許出願、又は第365条(c)項に基づく合衆国を指名したPCT国際出願の利益を主張し、本願の請求の範囲各項に記載の主題が合衆国法典第35部第112条第1項規定の態様で、先の合衆国特許出願又はPCT国際出願に開示されていない限度において、先の出願の出願日と本願の国内出願日又はPCT国際出願日の間に有効となった連邦規則法典第37部第1章第56条に記載の特許要件に所要の情報を開示すべき義務を有することを認める。

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.) (出願番号)	(Day/Month/Year Filed) (出願の年月日)	(現況) (特許済み、係属中　放棄済み)	(Status) (patented, pending, abandoned)
(Application No.) (出願番号)	(Day/Month/Year Filed) (出願の年月日)	(現況) (特許済み、係属中　放棄済み)	(Status) (patented, pending, abandoned)

☐ その他の合衆国又は国際特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional U.S. or international application numbers are listed on a supplemental priority sheet attached hereto.

私は、ここに自己の知識にもとずいて行った陳述がすべて真実であり、自己の有する情報および信ずるところに従って行った陳述が真実であると信じ、さらに故意に虚偽の陳述等を行った場合、合衆国法典第18部第1001条により、罰金もしくは禁錮に処せられるか、またはこれらの刑が併科され、またかかる故意による虚偽による陳述が本願ないし本願に対して付与される特許の有効性を損なうことがあることを認識して、以上の陳述を行ったことを宣言する。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

私、下記署名者は、ここに記載の米国弁護士または代理人に本出願に関し特許商標庁にて取られるいかなる行為に関して、同米国弁護士又は代理人が、私に直接連絡なしに私の外国弁護士或るいは法人代表者からの指示を受け取り、それに従うようここに委任する。この指示を出す者が変更の場合には、ここに記載の米国弁護士又は代理人にその旨通知される。

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from either his foreign patent agent or corporate representative, if any, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Japanese Language Utility or Design Patent Application Declaration

委任状： 私は、下記発明者として、下記に明記された顧客番号を伴う以下の弁護士又は、代理人をここに選任し、本順の手続きを遂行すること並びにこれに関する一切の行為を特許商標庁に対して行うことを委任する。そして全ての通信はこの顧客番号宛に発送される。

顧客番号 7055

現在選任された弁護士は下記の通りである。

POWER OF ATTORNEY: As a named inventor, I hereby appoint the attorney(s) and/or agent(s) associated with the Customer Number provided below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

CUSTOMER NUMBER 7055

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同発明者の署名 日付	Inventor's signature Date Susumu Seino June 29, 2000
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国籍	Citizenship Japan
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同第２共同発明者の署名 日付	Second Inventor's signature Date Tadao Shibasaki June 29, 2000
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国籍	Citizenship Japan
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（第六またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。）

(Supply similar information and signature for third and subsequent joint inventors.)

U.S. PTO Acceptance # 903

Japanese Language Utility or DesignPatent Application Declaration

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共同発明者の署名　日付	Third Inventor's signature　Date Nobuaki Ozaki June 28, 2000
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第四の共同発明者の氏名	Full name of fourth inventor, if any
共同発明者の署名　日付	Fourth Inventor's signature　Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address
第五の共同発明者の氏名	Full name of fifth inventor, if any
共同発明者の署名　日付	Fifth Inventor's signature　Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address
第六の共同発明者の氏名	Full name of sixth inventor, if any
共同発明者の署名　日付	Sixth Inventor's signature　Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address

(それ以降の共同発明者にたいしても同様な情報および署名を提供すること。)

(Supply similar information and signature for subsequent joint inventors.)

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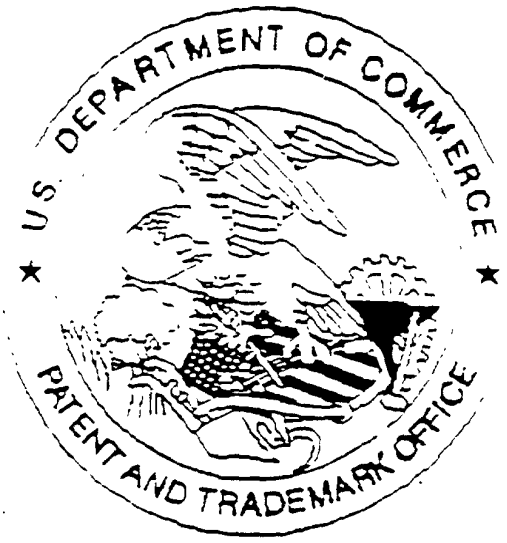

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